Infrared Spectra of *Bacillus subtilis* Spores: The Effect of Growth Media

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ABSTRACT

We present infrared microscopy spectra of *Bacillus subtilis* spores cultured in nine types of growth media, and also consider the effect of heat shock on the sporulation of the bacteria and the consequence on the infrared spectral properties of the whole microorganisms. The effect of growth media is elucidated with regard to the variance or invariance of several "marker bands" in the infrared spectrum of the microorganisms that can be used to effect detection and to some extent identification of the bacterial spores. We evaluate the degree of sporulation apparent in the various samples as a function of the growth media using optical microscopy, and compare the spectra of the samples to that of a sample consisting almost exclusively of vegetative cells. We use a correlation coefficient metric to explore the influence of growth media on detection models programmed using a sample of the microbial spores that is frequently used in field tests sponsored by the Department of Defense. While the results exhibit significant quantitative variation in the spectra as a function of growth media, we note that the qualitative properties of the spore spectra are preserved under the varying growing conditions.

INTRODUCTION

Infrared spectroscopy has proven value in environmental applications including the sensing and identification of chemical agents and hazardous air pollutants. Open-air monitoring of trace organic vapors is facilitated using Fourier transform open-path, retroreflector-based spectrometers, spectroradiometers and extractive gas cell-based sensors. In addition, infrared laser-induced detection and ranging (LIDAR) has been used for remote sensing of chemical plumes, relying on the spectral properties or "fingerprint" of the target analyte(s) in order to effect detection and identification. More recently there has been a significant level of interest in whether the infrared spectra of biological materials may afford a method for detection of hazardous biological materials in an analogous manner. A significant body of work is extant in the literature that demonstrates the utility of whole-organism infrared spectral analysis to the identification of bacteria and other microorganisms to the species and in some cases even the strain level[1-6]. Such studies are of significant interest to scientists seeking to develop advanced sensor technologies for chemical and biological defense applications. However, it is clear from the literature that extracting the spectral fingerprint of a particular microorganism from the infrared spectrum is not as straightforward as it is for organic liquids and gases because the basic chemical composition of all biological materials is largely independent of the species and strain. Strain-level identification has been demonstrated only under carefully controlled conditions of sample culturing, harvesting, and analysis[1-6]. Nevertheless, there continues to be a need for rapid environmental screening methods (e.g., aerosol monitors) that afford to some level of confidence the ability to distinguish between the ambient chemical and biomaterial composition and an anthropogenic release of a biological agent. We are interested in assessing the utility of infrared spectroscopy in just such a role. Our previous efforts have demonstrated that the infrared spectra of a sampling of several *Bacillus* bacteria do indeed present unique, repeatable, and to some extent peculiar fingerprints. A significant concern regarding the use of vibrational spectroscopic methods for biological sensing is the dependence of the observed apparent chemical composition of the prokaryote on the media and growth conditions used in preparing the sample[7]. We present herein a systematic study of this problem: we analyze the infrared spectra of

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Bacillus subtilis endospores that were cultured in a variety of liquid and solid growth media and assess the influence of media on three spectral regions that correspond to different molecular composition.

A typical infrared spectrum of *Bacillus subtilis* is presented in Figure 1 along with some annotation describing the principal molecular functionality represented by each of the observed vibrational bands. We note that the spectrum may be divided into five relatively distinct regions based on the measured molecular phenomena: (I) the hydroxyl, amide-A, and methyl/methylene bands dominate the spectrum in the shorter wavelength range (~3.3-3.6 microns), (II) the strong amide-I and amide-II, absorption bands (~5.6-6.7 microns), (III) the methylenene bend and carboxylate bands (~6.7-7.6 microns), and (IV) the phosphate/polysaccharide bands (~7.6-11 microns) present a rich structure in the mid infrared wavelength region, and (V) the so-called "fingerprint" region in the far infrared above 11 microns consists of weak but information-rich bands loosely attributed to aromatic constituents such as certain amino acids and nucleotides, and some methylene rocking modes exhibited by fatty acids[3]. We attempt to quantify the impact that the culture media has on the observable physical and biochemical properties of the *Bacillus* spores by studying the optical microscopy and infrared microspectroscopy of samples of the spores.

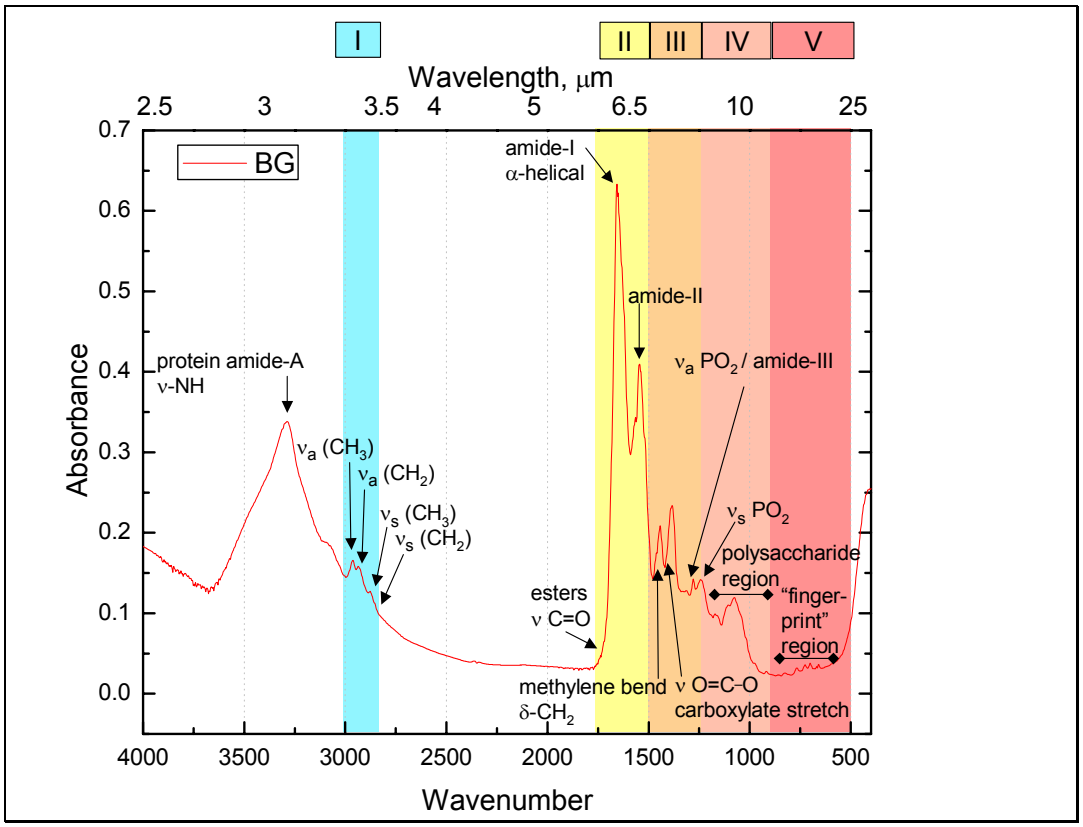

Figure 1. Annotated infrared spectrum of BG highlighting the five distinct regions used in this study.

METHODS

Bacillus subtilis var. niger was inoculated onto six different solid culture formulae: AK Agar #2 (AK)[8], Nutrient Sporulation Media (NSM)[9], Minimal Glucose Agar (MG)[9], Sporulation Agar (SA) (ATCC Culture Medium 5), Soil Extract Agar (SE) (ATCC Culture Medium 10 Nutrient Agar (Difco 0001) with 25% soil extract (ATCC Medium 191) in tap water), and Sphaericus Spore Medium (SS) (ATCC Culture Medium 1101). The bacteria were also inoculated into four different liquid broth formulae: Casein Acid Digest Medium (CAD)[10], NZ-Amine A (NZA)[10], Leighton-Doi Medium (LD)[11,12],

and NS broth (a liquid variety of the NSM agar). The cultures were checked daily (to a maximum period of 14 days) to evaluate the degree to which sporulation was achieved. The endospore suspensions were centrifuged directly out of the liquid media and resuspended in dDI water. In the case of the spores grown on solid media the bacteria were collected off of the agar using an inoculating paddle and suspended in distilled, deionozed (dDI) water. Some of the harvested cells were heat treated at 70-80º C for 20-30 minutes to evaluate the effect of heat shock on the sporulation efficiency. The suspensions were then repeatedly (3x) centrifuged and resuspended in fresh dDI water to wash away excess media, cellular material, etc., then lyophilized.

A sample of the lyophilized spores was again suspended in 18.3±0.1 MΩ ultrafiltered water from a Nanopure system, and vortexed (and sonicated as necessary in some cases) to form a relatively homogeneous suspension (approximately 1 milligram per milliliter). A 20 microliter aliquot of this suspension was deposited onto a gold-coated substrate prepared for infrared microanalysis as detailed in a related study[13]. A 10 microliter aliquot was air-dried onto a glass microscope slide for optical microscopy.

Optical micrographs were prepared using an Olympus BX40 optical/fluorescence microscope equipped with a CCD camera. The micrograph scale was calibrated using a 2-mm micrometer scale incremented to 10 micrometers. The microscope images were collected using the phase contrast setting on the stage illuminator to improve the resolution of the spores and ultraviolet top-illumination to accentuate the proteinaceous materials observed via their fluorescence properties.

Infrared microspectra were collected on a Digilab FTS7000 spectrometer/UMA600 infrared microscope equipped with an extended range MCT detector. Spectra are the result of an average of 128 single-sided scans collected at 4 cm^{-1} resolution and converted into frequency domain using triangular apodization. A 250x250 micron aperture setting was used to maximize the detector fill factor through the Cassegrainian objective. All spectra were acquired in reflectance-transmittance mode and ratioed to a background scan collected on a clean area of the gold-coated substrate. Six to twelve regions of the bacteria film were analyzed to produce a set of replicate spectra.

The replicate spectra for each sample were compared with the replicate spectra of a scrupulously washed BG sample prepared from a sporulating agar preparation[7]. We analyzed the spectra using a straightforward correlation metric implemented under Matlab™ (The Math Works, Inc.). The purpose of the analysis was simply to evaluate the degree to which the spectra of the spores grown under the various media correlated with the control set (the sample prepared from the sporulating agar). The spectra preprocessed by taking the first derivative employing a three-point smoothing function to reduce baseline and measurement noise effects. For the purposes of this analysis five spectral regions were studied independently: region I, 2800-3000 cm^{-1}, region II, 1500-1800 cm^{-1}, region III, 1300-1500 cm^{-1}, region IV, 900-1300 cm^{-1}, and region V, 500-900 cm^{-1}. In order to evaluate the correlation between the i^{th} spectrum, $\alpha_i(\lambda)$, of each sample to a j^{th} reference spectrum (the BG control sample), $\alpha_j^{ref}(\lambda)$, we computed a linear correlation coefficient $r(\alpha_i, \alpha_j^{ref})$ using the following relationship:

$$r(\alpha_i, \alpha_j^{ref}) = \frac{\sum_{\lambda_{\min}}^{\lambda_{\max}} [\alpha_i(\lambda) - E(\alpha_i(\lambda))][\alpha_j^{ref}(\lambda) - E(\alpha_j^{ref}(\lambda))]}{\sigma_{\alpha_i}\sigma_{\alpha_j^{ref}}},$$

where $E(\alpha)$ and σ_α are the mean and standard deviation of α within the wavenumber range $\lambda_{\min}$ to $\lambda_{\max}$. From the six replicate spectra in each measurement set we computed 36 values of the correlation coefficient (*i* and *j* takes the value of 1 to 6), all of which are plausible correlations due to the different combinations (*i, j*). The mean linear correlation between a sample and the BG control sample is $r(sample, ref) = E(r(i, j))$ and the standard deviation of the 36 correlation values reflects the spectral similarity between a measurement set of BG grown in a given growth medium to the BG control and the uncertainty due to the sample preparation procedure.

RESULTS

The raw infrared microspectra of each sample is shown as a function of growth media in Figures 2-6. Also shown in the figures are the micrographs collected using the UMA600 microscope of a representative area of the actual microsamples. The spores appear in these images as a section of a circular film (we chose an area of the sample near the film edge to create the micrographs, so that the film and the gold surface can be seen together in the images), and the central square defined by the rectangular grid represents the aperture setting (a 250x250 micron square). The micrographs reveal extraneous material in most of the films besides the BG control sample. A particularly striking case is seen in the image of the LD sample (Figure 3). The presence of the extraneous material manifests to a large extent as an apparent shift in baseline and/or magnitude in the replicate measurements as can be clearly seen in the accompanying spectral plots. The results are somewhat subjective in that regions of the film that exhibited gross nonuniformity or damage were generally avoided during the data collection.

A special case sample was that prepared using the sporulation agar (SA). The SA medium failed to produce a high yield of spores, and it was concluded that the majority of the material recovered from the agar consisted largely of vegetative cells. The lyophilized material could be described as a spongy mass, and was difficult to resuspend in water. We prepared two samples from the material. In one case, we merely vortexed the suspension for several minutes and performed the microspectroscopy on the relatively large sheets of resulting material as seen in Figure 6. The second SA sample was sonicated for several minutes to afford a more uniform (but still very inhomogeneous) suspension, and performed the microspectroscopy on the more uniform regions of the film, avoiding the fragments of material (Figure 6). We had hoped that this approach would afford a comparison between the spectra of the spores and that of the cellular material.

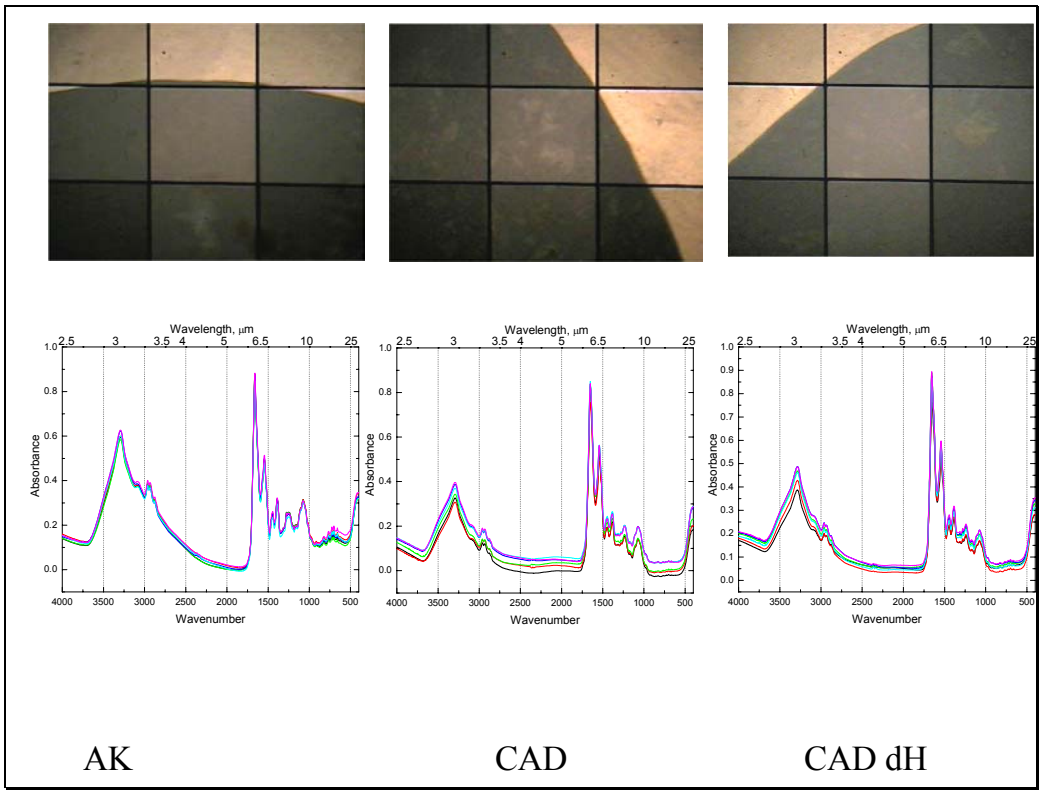

Figure 2. Optical micrographs and corresponding infrared spectra of films of BG grown on AK and CAD media. The dH annotation denotes the spores that were heat treated at 70 ºC.

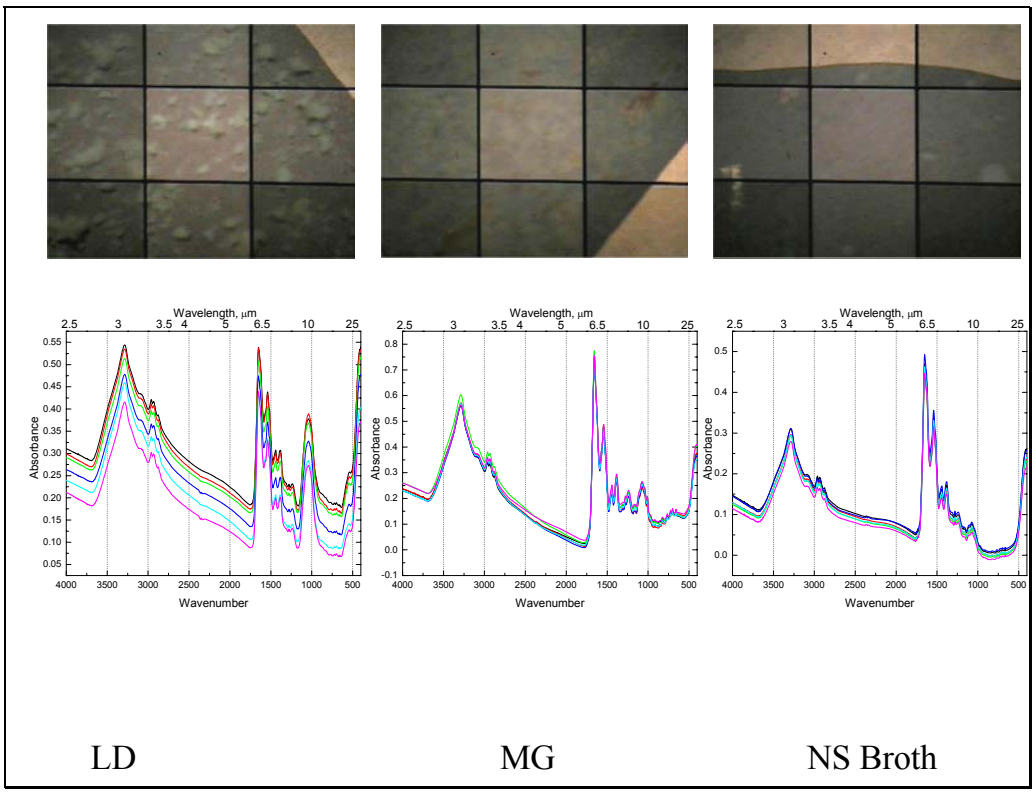

Figure 3. Optical micrographs and corresponding infrared spectra of films of BG grown on LD, MG and NS broth media.

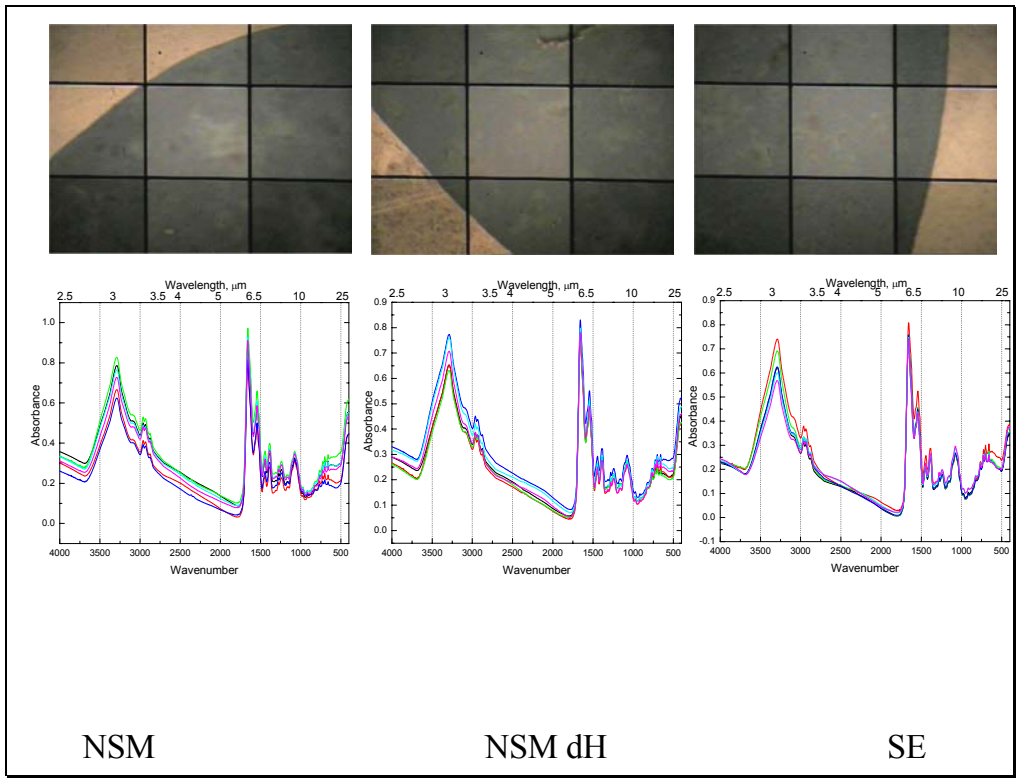

Figure 4. Optical micrographs and corresponding infrared spectra of films of BG grown on NSM and SE media. The dH annotation denotes the spores that were heat treated at 70 ºC.

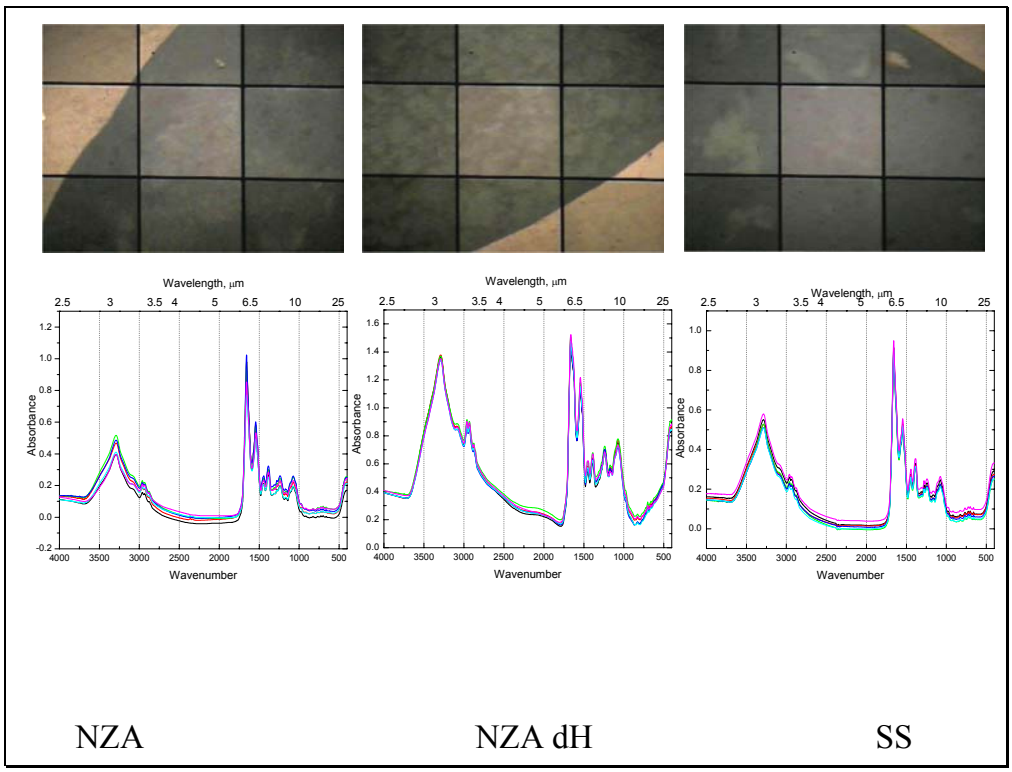

Figure 5. Optical micrographs and corresponding infrared spectra of films of BG grown on NZA and SS media. The dH annotation denotes the spores that were heat treated at 70 ºC.

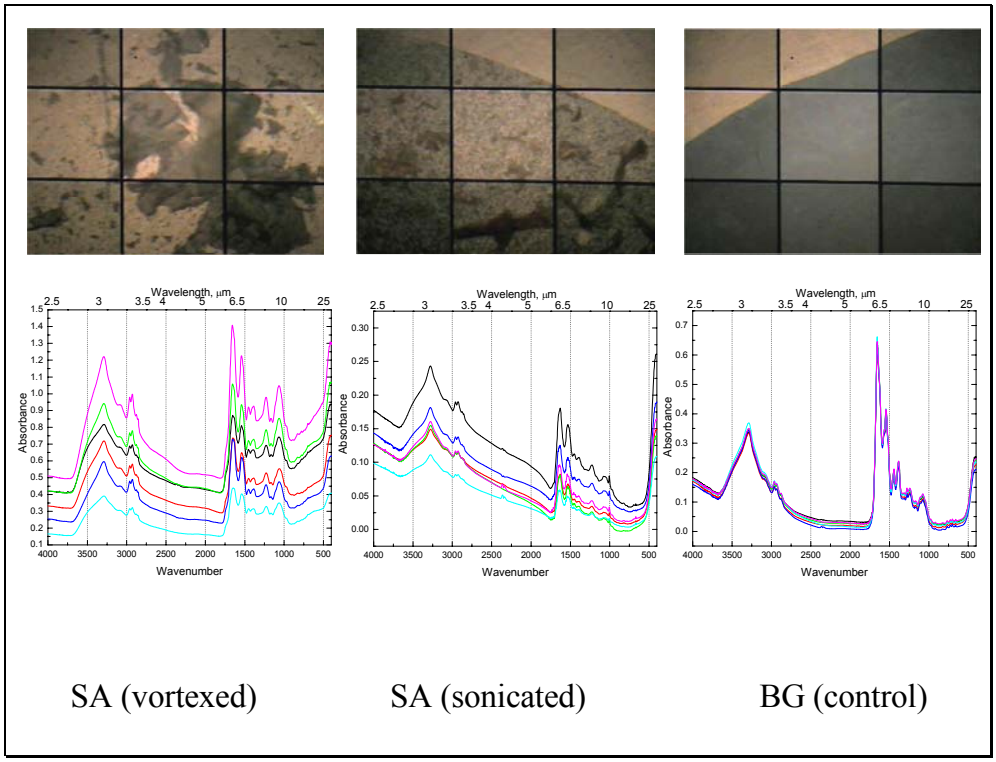

Figure 6. Optical micrographs and corresponding infrared spectra of films of BG grown on SA media, treated by vortexing and sonication, and of the control sample.

The results of our correlation analysis for each of the five wavelength ranges are shown in Figure 7. It is apparent in Figure 7 that region V (the fingerprint region) is more sensitive to the media on which the spores were cultured than is any of the other regions (I – IV) studied, and there is a clearly indicated trend towards greater sensitivity to growth media with increasing wavelength. In region I, essentially no correlation was observed except as expected in the case of the SA samples. In regions II and III, the AK, LD, and heat-treated NZA samples begin to exhibit spectral effects that are apparently correlated to the growth medium. Otherwise, the correlation with media did not appear to be significant. In the region IV, the MG sample appears to exhibit significant correlation with media while the AK sample does not. In region V only the CAD, NS broth, and SS preparations exhibited negligible correlation with media as compared with the control sample; very significant correlation with growth media was apparent in all of the other samples, particularly in the case of AK, LD, MG, NSM, and SE. Noteworthy in the region II-V data is the large observed effect of heat treatment in the case of the NZA media only. The degree of spectral correlation with media increased in the case of the heat-treated spores grown in the NZA medium versus the untreated spores grown in the same medium. Heat treatment as applied to the spores grown on CAD and NSM media did not have as significant an effect on the spectral properties of the samples. The apparent quantity of extraneous material was larger in the case of the NZA sample; it is uncertain whether this is an effect of the heat treatment itself or an artifact of sample handling.

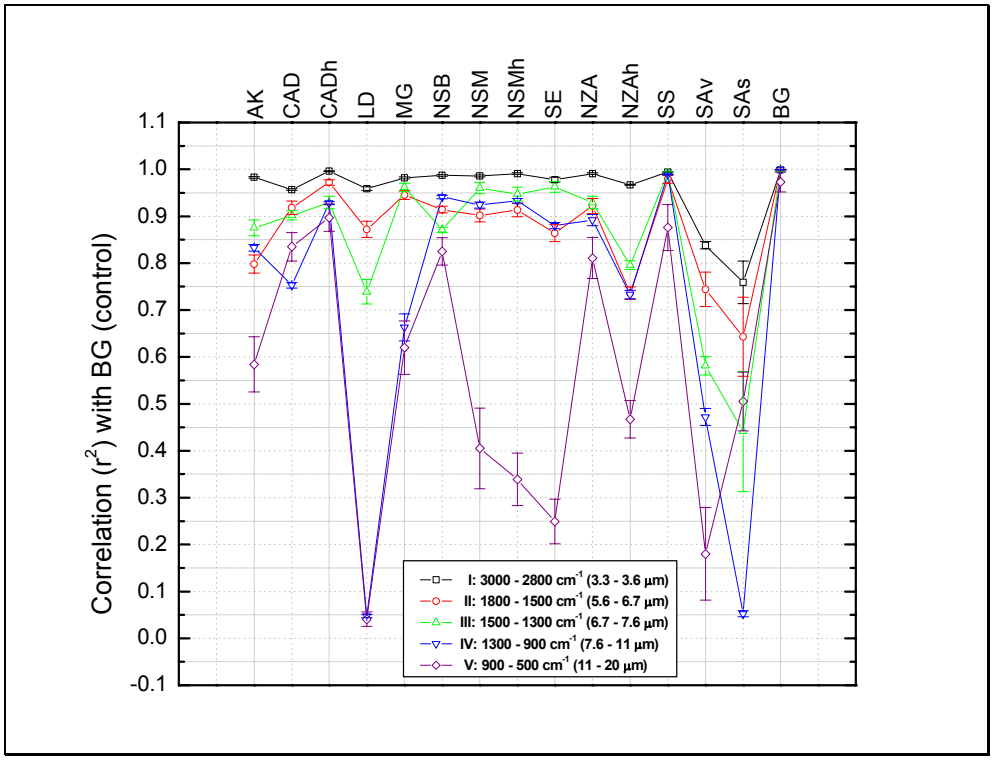

Figure 7. Linear correlation results from the analysis of spectra of the BG grown under each type of media and sample treatment protocol compared to the control sample as described in the text.

CONCLUSIONS

The purpose of this study was to explore the effect of growth media on the spectral properties of the BG material, and to draw some conclusions about the effect that variations due to growth media may have on detection models based on a "standard" BG spectrum. It would be easy to over-interpret the results presented herein. It is clear from the microscope images of the measured samples that much of the variability observed in the spectra may in fact be due to extraneous, non-spore material associated with

the sample. Possible sources of the materials may include cellular by-products, mother cell components, and residual media. The spores cultured in the LD media exhibited the most dramatic abundance of extraneous material as can be clearly seen in the micrograph of Figure 3. In an effort to elucidate the nature of this extraneous material, we examined the sample using bright field optical and phase contrast microscopy and dark field fluorescence microscopy. The extraneous material exhibited bright fluorescence, suggesting that it may be proteinaceous. Similar results were observed to a varying degree in all of the BG samples including the control, but generally to a much lesser extent.

A clear implication of the results is that particularly in the longer wavelengths, the IR spectra are sensitive to the effect of culture media on the samples. It is less clear whether the effect is a direct result of extraneous material or intrinsic to the bacterial spores.

We conclude that the growth media and sample processing may result in significant quantitative variation of the observed infrared spectra of the spores, particularly in the 500-900 wavenumber spectral region. However, the qualitative spectra of the bacterial spores are preserved despite the differences in growth media, and the differences that are observed may be more dependent on the presence of extraneous, non-spore material that accompanies the samples as a function of growth media and sample handling than on the intrinsic properties of the spores themselves. We note that in early studies of staphylococcal isolates grown on peptone agar vs. blood agar[14], cluster analysis on the infrared spectra failed to resolve the effect of the medium; however in a later study comparing staphylococcal isolates grown on TY agar to those grown on blood agar, a similar analysis distinguished between the two samples[7]. A noteworthy difference in the infrared measurements made in these two studies vis-à-vis those performed in this study is the low wavenumber limit in the early studies was limited to 700 cm^{-1}. The region between 500 and 700 cm^{-1} is expected to be important in the analysis of bacterial spores because of their picolinic acid content. We are engaged in further studies to elucidate this problem and to evaluate the utility of the infrared spectra in the discrimination between bacterial spores and other biological materials.

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